The Examiner has made Claims 1-20 subject to a restriction requirement under 35 U.S.C. 121. The Examiner has divided Claims 1-20 into eight groups, a summary of which follows:

I. Claims 1, 8, 9, 12, 19 limited to subgenera G1 and G3;

II. Claims 1-6, 8, 12-16, 19 limited to subgenera G1 and G4;

III. Claims 1, 8, 9, 12, 19 limited to subgenera G2 and G3;

IV. Claims 1-6, 8, 12-16, 19 limited to subgenera G2 and G4;

V. Claims 17-18, drawn to a method of using the compounds of Group I;

VI. Claims 17-18, drawn to a method of using the compounds of Group II;

VII. Claims 17-18, drawn to a method of using the compounds of Group III; and

VIII. Claims 17-18 drawn to a method of using the compounds of Group IV.

Restriction is only proper if the restricted inventions are independent and patentably distinct (35 USC §121) and there is a *serious burden* placed on an Examiner if restriction is not required (MPEP 803). The burden is on an Examiner to provide reasons and/or examples to support any conclusions of patentable distinctness between the restricted inventions (MPEP 803). Applicants respectfully traverse the Restriction Requirement on the grounds that no adequate reasons and/or examples have been provided to support a conclusion of patentable distinctness between the restricted inventions and that no serious burden is placed on the Examiner if restriction is not required.

For the above reasons, Applicants submit that the restriction by the Examiner is improper and request that the restriction be withdrawn or reconsidered.

Respectfully submitted,

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UTILITY

The compounds of Formula (I) are expected to inhibit the activity of Hepatitis C Virus NS3 protease. The NS3 protease inhibition is demonstrated using assays for NS3 protease activity, for example, using the assay described below for assaying inhibitors of NS3 protease. The compounds of Formula (I) are expected to show activity against NS3 protease in cells, as demonstrated by the cellular assay described below. Thus, the compounds of Formula (I) are potentially useful in the cure and prevention of HCV infections.

Expression and Purification of NS3 Protease

The plasmid cf1SODp600, containing the complete coding region of HCV NS3 protease, genotype 1a, was obtained from ATCC (database accession: DNA Seq. Acc. M62321, originally deposited by Chiron Corporation). PCR primers were designed that allow amplification of the DNA fragment encoding the NS3 protease catalytic domain (amino acids 1 to 192) as well as its two N-terminal fusions, a 5 amino acid leader sequence MGAQH **(SEQ. ID. NO.:1)** (serving as a expression tag) and a 15 amino acid His tag MRGSHHHHHHMGAQH **(SEQ. ID. NO.:2)**. The NS3 protease constructs were cloned in the bacterial expression vector under the control of the T7 promoter and transformed in *E. coli* BL 21 (DE3) cells. Expression of the NS3 protease was obtained by addition of 1 mM IPTG and cells were growing for additional 3h at 25°C. The NS3 protease constructs have several fold difference in expression level, but exhibit the same level of solubility and enzyme specific activity. A typical 10 L fermentation yielded approximately 200 g of wet cell paste. The cell paste was stored at -80°C. The NS3 protease was purified based on published procedures (Steinkuhler C. et al. *Journal of Virology* 70, 6694-6700, 1996 and Steinkuhler C. et al. *Journal of Biological Chemistry* 271, 6367-6373, 1996.) with some modifications. Briefly, the cells were

resuspended in lysis buffer (10 mL/g) containing PBS buffer (20 mM sodium phosphate, pH 7.4, 140 mM NaCl), 50% glycerol, 10 mM DTT, 2% CHAPS and 1mM PMSF. Cell lysis was performed with use of microfluidizer. After homogenizing, DNase was added to a final concentration 70 U/mL and cell lysate was incubated at 4°C for 20 min. After centrifugation at 18,000 rpm for 30 min at 4°C supernatant was applied on SP Sepharose column (Pharmacia), previously equilibrated at a flow rate 3 mL/min in buffer A (PBS buffer, 10% glycerol, 3 mM DTT). The column was extensively washed with buffer A and the protease was eluted by applying 25 column volumes of a linear 0.14 - 1.0 M NaCl gradient. NS3 containing fractions were pooled and concentrated on an Amicon stirred ultrafiltration cell using a YM-10 membrane. The enzyme was further purified on 26/60 Superdex 75 column (Pharmacia), equilibrated in buffer A. The sample was loaded at a flow rate 1 mL/min, the column was then washed with a buffer A at a flow rate 2 mL/min. Finally, the NS3 protease containing fractions were applied on Mono S 10/10 column (Pharmacia) equilibrated in 50 mM Tris.HCl buffer, pH 7.5, 10% glycerol and 1 mM DTT and operating at flow rate 2 mL/min. Enzyme was eluted by applying 20 column volumes of a linear 0.1 - 0.5 M NaCl gradient. Based on SDS-PAGE analysis as well as HPLC analysis and active site titration, the purity of the HCV NS3 1a protease was greater than 95%. The enzyme was stored at -70°C and diluted just prior to use.

Enzyme Assays

Concentrations of protease were determined in the absence of NS4a by using the peptide ester substrate Ac-DED(Edans)EEAbuψ[COO]ASK(Dabcyl)-NH_2 **(SEQ. ID. NO.:3)** (Taliani et al. *Anal. Biochem.* 240, 60-67, 1996.) and the inhibitor, H-Asp-Glu-Val-Val-Pro-boroAlg-OH **[and the inhibitor, H-Asp-Glu-Val-Val-Pro-boroAlg-OH] (SEQ. ID. NO.:5)** and by using tight binding reaction conditions (Bieth, *Methods Enzymol.* 248, 59-85, 1995). Best data was

obtained for an enzyme level of 50 nM. Alternately, protease (63 μg/mL) was allowed to react with 3 μM NS4a, 0.10 mM Ac-Glu-Glu-Ala-Cys-*p*NA **(SEQ. ID. NO.:4)**, and varying level of H-Asp-Glu-Val-Val-Pro-boroAlg-OH (0-6 μM). Concentrations of protease were determined from linear plots of Activity vs.[inhibitor]. Molar concentrations of proteases were determined from the x-intercept.

K_m values were determined measuring the rate of hydrolysis of the ester substrate over a range of concentrations from 5.0 to 100 μM in the presence of 3 μM KKNS4a (KKGSVVIVGRIVLSGKPAIIPKK) **(SEQ. ID. NO.:6)**. Assay were run at 25°C, by incubating ~1 nM enzyme with NS4a for 5 min in 148 μl of buffer (50 mM Tri buffer, pH 7.0, 50% glycerol, 2% Chaps, and 5.0 mM DTT. Substrate (2.0 μl) in buffer was added and the reaction was allowed to proceed for 15 min. Reactions were quenched by adding 3.0 μL of 10% TFA, and the levels of hydrolysis were determined by HPLC. Aliquots (50 μL) were injected on the HPLC and linear gradients from 90% water, 10% acetonitrile and 0.10 % TFA to 10% water, 90% acetonitrile and 0.10% TFA were run at a flow rate of 1.0 mL/min over a period of 30 min. HPLCs were run on a HP1090 using a Rainin 4.6 x 250 mm C18 column (cat # 83-201-C) fluorescent detection using 350 and 500 nm as excitation and emission wavelengths, respectively. Levels of hydrolysis were determined by measuring the area of the fluorescent peak at 5.3 min. 100% hydrolysis of a 5.0 μM sample gave an area of 7.95 ±0.38 fluorescence units.). Kinetic constants were determined from the iterative fit of the Michaelis equation to the data. Results are consistent with data from Liveweaver Burk fits and data collected for the 12.8 min peak measured at 520 nm.

Enzyme activity was also measured by measuring the increase in fluorescence with time by exciting at 355 nm and measuring emission at 495 nm using a Perkin Elmer LS 50

spectrometer. A substrate level of 5.0 μM was used for all fluorogenic assays run on the spectrometer.

Inhibitor Evaluation *In vitro*

Inhibitor effectiveness was determined by measuring enzyme activity both in the presence and absence of inhibitor. Velocities were fit to the equation for competitive inhibition for individual reactions of inhibitors with the enzyme using

$$v_i / v_o = [K_m (1 + I/K_i) + S] / [K_m + S].$$

The ratio v_i / v_o is equal to the ratio of the Michaelis equations for velocities measured in the presence (v_i) and absence (v_o) of inhibitor. Values of v_i / v_o were measured over a range of inhibitor concentrations with the aid of an Excel™ Spreadsheet. Reported K_i values are the average of 3-5 separate determinations. Under the conditions of this assay, the IC_{50} and K_is are comparable measures of inhibitor effectiveness.

Using the methodology described above, compounds of the present invention were found to exhibit a K_i of ≤60 μM, thereby confirming the utility of the compounds of the present invention as effective NS3 protease inhibitors. Preferred compounds of the present invention have K_i's of ≤1 μM. More preferred compounds of the present invention have K_i's of ≤100 nM. Most preferred compounds of the present invention have K_i's of ≤10 nM.

Inhibitor Evaluation in Cell Assay.

The following method was devised to assess inhibitory action of test compounds on the HCV NS3 protease in cultured cells. Because it is not possible to efficiently infect cells with hepatitis C virus, an assay was developed based on co-expression in transfected cell lines of two plasmids, one is able to direct synthesis of the NS3 protease and the other to provide a polypeptide analogous to a part of the HCV non-structural protein containing a single known peptide sequence highly susceptible to

cleavage by the protease. When installed in cultured cells by one of a variety of standard methods, the substrate plasmid produces a stable polypeptide of approximately 50KD, but when the plasmid coding for the viral protease is co-expressed, the enzymatic action of the protease hydrolyzes the substrate at a unique sequence between a cysteine and a serine pair, yielding products which can be detected by antibody-based technology, eg, a western blot. Quantitation of the amounts of precursor and products can be done by scanning film auto-radiograms of the blots or direct luminescense-based emissions from the blots in a commercial scanning device. The general organization of the two plasmids is provided in Scheme 6. The coding sequences for the NS3 protease and the substrate were taken from genotype 1a of HCV, but other genotypes, eg 2a, may be substituted with similar results.

The DNA plasmids are introduced into cultured cells using electroporation, liposomes or other means. Synthesis of the protease and the substrate begin shortly after introduction and may be detected within a few hours by immunological means. Therefore, test compounds are added at desired concentrations to the cells within a few minutes after introducing the plasmids. The cells are then placed in a standard CO_2 incubator at 37°C, in tissue culture medium eg Dulbecco-modified MEM containing 10% bovine serum. After 6-48 hours, the cells are collected by physically scraping them from plastic dishes in which they have been growing, centrifuging them and then lysing about 10^6 of the concentrated cells in a minimal volume of buffered detergent, eg 20 µl of 1% sodium dodecyl sulfate in 0.10 M Tris-HC1, pH 6.5, containing 1% mercaptaethanol and 7% glycerol. The samples are then loaded onto a standard SDS polyacrylamide gel, the polypeptides separated by electrophoresis, and the gel contents then electroblotted onto nitrocellulose or other suitable paper support, and the substrate and products detected by decoration with specific antibodies.

Although this invention has been described with respect to specific embodiments, the details of these embodiments are not to be construed as limitations. Various equivalents, changes and modifications may be made without departing from the spirit and scope of this invention, and it is understood that such equivalent embodiments are part of this invention.

Preparation of H-Asp-Glu-Val-Val-Pro-boroAlg pinanediol ester•trifluoroacetate **(SEQ. ID. NO.:7)**.

Preparation of Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH **(SEQ. ID. NO.:8)**. Boc-Val-Pro-OBzl was prepared by dissolving H-Pro-OBzl (20 g, 83 mmol) in 50 mL of chloroform and adding Boc-Val-OH (18.0 g, 83 mmol), HOBt (23.0g, 165 mmol), NMM (9.0 mL, 83 mmol) and DCC (17.0 g, 83 mmol). The reaction mixture was stirred overnight at room temperature. The mixture was filtered and solvent was evaporated. Ethyl acetate was added and insoluble material was removed by filtration. The filtrate was washed with 0.2N HCl, 5% $NaHCO_3$, and saturated aqueous NaCl. It was dried over Na_2SO_4, filtered and evaporate to give a white solid (30 g, 75 mmol, 90%). ESI/MS calculated for $C_{22}H_{32}N_2O_5$ +H: 405.2. Found 405.6.

Boc-Val-Val-Pro-OBzl was prepared by dissolving Boc-Val-Pro-OBzl (14.0 g, 35.0 mmol) in 4N HCl in dioxane (20 mL) and allowing the reaction to stir for 2h under an inert atmosphere at room temperature. The reaction mixture was concentrated by evaporation *in vacuo* and ether was added to yield a precipitate. It was collected by filtration under nitrogen. After drying *in vacuo* with P_2O_5, H-Val-Pro-OBzl was obtained as a white solid (22.6 g, 30.3 mmol, 89%). (ESI/MS calculated for $C_{17}H_{24}N_2O_3$ +H: 305.2. Found: 305.2.) H-Val-Pro-OBzl (9.2 g, 27 mmol) was dissolved in 50 mL of CH_2Cl_2 and Boc-Val-OH (7.3 g, 27 mmol), HOBt (7.3 g, 54 mmol), NMM (3.0 mL, 27 mmol) and DCC (5.6 g, 27 mmol)

were added. The reaction mixture stirred overnight at room temperature. The mixture was filtered and the filtrate was evaporated. The residue was dissolved in ethyl acetate and the solution was re-filtered. The filtrate was washed with 0.2N HCl, 5% $NaHCO_3$, and saturated aqueous NaCl. It was dried over Na_2SO_4, filtered and evaporated to give a yellow oil (10.6 g, 21.1 mmol, 78%). ESI/MS calculated for $C_{27}H_{41}N_3O_6$ + Na: 526.3 Found: 526.4.

Z-Glu(O^tBu)-Val-Val-Pro-OBzl **(SEQ. ID. NO.:9)** was also prepared by DCC coupling. H-Val-Val-Pro-OBzl•hydrochloride was obtained in a 100% yield by treating the corresponding Boc compound with anhydrous HCl using the procedure described for H-Val-Pro-OBzl (ESI/MS calculated for $C_{22}H_{33}N_3O_4$ + H: 404.2. Found 404.3.). The amine hydrochloride (7.40 g, 16.8 mmol) was dissolved in 185 mL DMF and 25 mL THF. Z-Glu(O^tBu)-OH (5.60 g, 16.8 mmol), HOBt (4.60 g, 33.6 mmol), NMM (1.85 mL, 16.8 mmol) and DCC (3.5 g, 16.8 mmol) were added. The reaction was run and the product was isolated by the procedure described for Boc-Val-Val-Pro-OBzl. The tetrapeptide was obtained as a white foam (12.0 g, 16.1 mmol, 96%). ESI/MS calculated for $C_{39}H_{54}N_4O_9$ + Na: 745.4. Found: 745.4.

H-Glu(O^tBu)-Val-Val-Pro-OH **(SEQ. ID. NO.:10)** was prepared by dissolving Z-Glu(O^tBu)-Val-Val-Pro-OBzl (2.90 g, 3.89 mmol) in 100 mL methanol containing 1% acetic acid. Pearlman's catalyst, $Pd(OH)_2$, (100mg) was added and the flask was placed on the Parr hydrogenation apparatus with an initial H_2 pressure of 34 psi. After three hours, the catalyst was removed by filtration through a celite pad and the filtrate was evaporated *in vacuo* to yield a yellow oil (1.30 g, 2.61 mmol, 67%). ESI/MS calculated for $C_{24}H_{42}N_4O_7$ +H: 499.3 Found: 499.4.

Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH was prepared by active ester coupling. Boc-Asp(O^tBu)-N-hydroxysuccinimide

ester was prepared by coupling Boc-Asp(OtBu)-OH (3.00 g, 10.4 mmol) to N-hydroxysuccinimide (1.19 g, 10.4 mmol) in 50 mL of ethylene glycol dimethyl ether. The reaction flask was placed in an ice bath at 0°C and DCC was added. The reaction mixture was slowly allowed to warm to room temperature and to stir overnight. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and re-filtered. The filtrate was evaporated give a white solid. Recrystallized from ethyl acetate: hexane gave the activated ester (3.38 g, 8.80 mmol, 84%). (ESI/MS calculated for $C_{17}H_{26}N_2O_8$ + H: 387.2. Found: 387.4.) H-Glu(O^{t}Bu)-Val-Val-Pro-OH (5.40 g, 10.8 mmol) was dissolved in 100 mL of water. Sodium bicarbonate (0.92 g, 11.0 mmol) was added followed by triethylamine (2.30 mL, 16.5 mmol). The N-hydroxysuccinimide ester (3.84 g, 10.0 mmol) was dissolved in 100 mL dioxane and was added to the H-Glu(O^{t}Bu)-Val-Val-Pro-OH solution. The mixture stirred overnight at room temperature. Dioxane was removed *in vacuo* and 1.0 M HCl was added to give pH ~ 1. The product was extracted into ethyl acetate. The ethyl acetate solution was washed with 0.2 N HCl, dried over sodium sulfate, filtered, and evaporated to yield a yellow oil (7.7 g, 10.0 mmol, 100%). ESI/MS calculated for $C_{37}H_{63}N_5O_{12}$ + Na: 792.4. Found: 792.4.

Boc-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Val-Pro-boroAlg-pinanediol **(SEQ. ID. NO.:11)** was prepared by coupling the protected pentapeptide to H-boroAlg-pinanediol. Boc-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Val-Pro-OH (1.8 g, 2.3 mmol) was dissolved 10 mL THF and was cooled to -20°C. Isobutyl chloroformate (0.30 mL, 2.3 mmol) and NMM (0.25 mL, 2.3 mmol) were added. After 5 minutes, this mixture was added to H-boroAlg-pinanediol (0.67 g, 2.3 mmol) dissolved in THF (8 mL) at -20°C. Cold THF (~5 mL) was used to aid in the transfer. Triethylamine (0.32 mL, 2.3 mmol) was added and the reaction mixture was allowed to come to room temperature

and to stir overnight. The mixture was filtered and solvent was removed by evaporation. The residue was dissolved in ethyl acetate, washed with 0.2 N HCl, 5% $NaHCO_3$, and saturated NaCl. The organic phase was dried with Na_2SO_4, filtered, and evaporated to yield a yellow oil. Half of the crude product (1.5 g) was purified in 250 mg lots by HPLC using a 4 cm x 30 cm Rainin C-18 reverse phase column. A gradient from 60: 40 acetonitrile: water to 100% acetonitrile was run over a period of 28 minutes at a flow rate of 40 mL/min. The fractions containing the desired product were pooled and lyophilized to yield a white solid (46 mg). ^{1}H-NMR (CD_3OD) δ 0.9-1.0 (m, 15H), 1.28 (s, 3H), 1.3 (s,3H), 1.44 (3s, 27H), 1.6-2.8 (20H), 3.7(m,1H), 3.9(m, 1H), 4.1-4.7 (7H), 5.05(m, 2H), 5.9(m, 1H). High res (ESI/MS) calculated for $C_{51}H_{86}N_6O_{13}B_1$ +H: 1001.635. Found 1001.633.

Preparation of H-Asp-Glu-Val-Val-Pro-boroAlg-pinanediol ester•trifluoroacetate: The hexapeptide analog, Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-boroAlg-pinanediol, (22.5 mg, 0.023 mmol) was treated with 2 mL of TFA: CH_2Cl_2 (1: 1) for 2 h. The material was concentrated *in vacuo* and purified by HPLC using C-18 Vydac reverse phase (2.2 x 25 cm) column with a gradient starting at 60:40 acetonitrile/water with 0.1%TFA going to 95:5 over 25 minutes with a flow rate of 8 mL/min. The product eluted at 80% acetonitrile. The fractions were evaporated and dried under high vacuum to give 8.9 mg (49%) of the desired product as white amorphous solid. ^{1}H-NMR (CD_3OD) δ 5.82 (m, 1H), 5.02 (m, 2H), 4.58(m, 1H), 4.42 (m, 3H), 4.18 (m, 4H), 3.90 (m, 1H), 3.62 (m, 1H), 3.01 (dd, 1H), 2.78 (m, 1H), 2.62 (m, 1H), 2.41-1.78 (m, 17H), 1.31 (s, 3H), 1.28 (s, 3H), 1.10 - 0.82 (m, 15H). ESI/MS calculated for $C_{38}H_{62}N_6O_{11}B$ +H: 789.2. Found: 789.2.